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Antibody Responses to an Immunodominant Nonstructural 1 Synthetic Peptide in Patients With Dengue Fever and Dengue Hemorrhagic Fever

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Two flaviviruses, dengue (DEN) virus and Japanese encephalitis (JE) virus, are important because of their global distribution and the frequency of epidemics in tropical and subtropical areas. To study the B-cell epitopes of nonstructural 1 (NS1) glycoprotein and anti-NS1 antibody response in DEN infection, a series of 15-mer synthetic peptides from the predicted B-cell linear epitopes of DEN-2 NS1 protein were prepared. Enzyme-linked immunosorbent assay (ELISA) was performed to analyze antibody responses to these peptides from sera of both DEN and JE patients. One peptide derived from DEN-2 NS1, D2 NS1-P1 (amino acids 1–15), was identified as the immunodominant epitope that reacted with sera from dengue fever (DF) patients but not JE patients. The isotype of D2 NS1-P1-specific antibodies was mainly immunoglobulin M (IgM) in all sera that tested positive. A specificity study demonstrated that sera from all four DEN types reacted with D2 NS1-P1. A dynamics study showed that specific antibodies to this peptide could be detected as early as 2 days after the onset of symptoms. We observed significant anti-D2 NS1-P1 antibody responses in 45% of patients with primary and secondary infections with DF or with dengue hemorrhagic fever. This is the first report demonstrating that significant anti-DEN NS1 antibodies can be induced in the sera of patients with primary DEN infection. ***J. Med. Virol. 57:1–8, 1999.***

KEY WORDS: dengue; nonstructural 1; synthetic peptide

INTRODUCTION

The family *Flaviviridae* consists of many important human pathogens. Among various subgroups, dengue (DEN) virus and Japanese encephalitis (JE) virus are the most prevalent in tropical and subtropical regions of the world. The global increase of morbidity and mortality of DEN virus infections has grown dramatically in recent decades. The disease is now endemic in more than 100 countries in Africa, the Americas, the Eastern Mediterranean, South-East Asia, and the Western Pacific [Gubler, 1997]. Although most DEN virus infections cause minimal symptoms or uncomplicated dengue fever (DF) [Burke et al., 1988], severe and life-threatening forms of the disease, dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS), occur in a small percentage of DEN infections [Nimmannitya et al., 1969; World Health Organization, 1986, 1994; Nimmannitya, 1987]. There are four distinct dengue viruses, types 1–4, and recovery from one DEN virus infection will not provide protection against subsequent infection by the other three. Indeed, there is compelling evidence to suggest that infection by a second DEN virus will actually increase the risk of more severe disease, resulting in DHF/DSS.

Understanding the pathogenesis of DHF is one of the most important subjects in DEN virus research. Although antibodies to DEN virus have been shown to mediate neutralization activities in *in vitro* assay [Russell et al., 1967], they can also augment DEN virus infection through Fcγ receptor-positive cells, such as monocytes and macrophages [Halstead and O'Rourke, 1977; Mady et al., 1993]. Halstead [1970] first hypothesized that DHF/DSS is associated with antibody-dependent enhancement (ADE) of virus replication

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from a previous heterologous DEN infection. Accumulated data from epidemiological, pathologic, clinical, and immunological studies strongly support this hypothesis and suggest that most cases of DHF are caused by immunopathologic mechanisms [Halstead et al., 1973; Halstead, 1979, Sangkawibha et al., 1984; Nimmannitya, 1987; Burke et al., 1988; Bharmarapravati, 1989]. Because some patients develop DHF during primary DEN infection without having preexisting DEN-specific antibodies, other factors, including the viral virulence [Rosen, 1977; Gubler et al., 1978] and the host genetic background [Bravo et al., 1987], may also contribute to the pathogenesis of DHF/DSS.

To develop a safe and effective DEN vaccine, a better understanding of the immunological response of infected individuals to various viral proteins and their roles in protective immunity and/or pathogenesis of DHF is greatly needed. Accordingly, it is important to analyze the immunological responses to various structures and nonstructural (NS) proteins and to distinguish between protective and pathogenic immunities.

In this study, we intended to identify continuous, immunodominant, B-cell epitopes on the NS1 protein of DEN virus by using synthetic peptides and to analyze the antibody responses to these peptides in the patients with DF and DHF. The results demonstrate that a DEN NS1-specific peptide, D2 NS1-P1, is identified. Antibodies to this peptide can be detected in the sera of patients with DF and DHF. We did not observe direct correlation between anti-NS1 antibodies and DHF, because sera from patients with DF and DHF showed similar anti- D2 NS1-P1 antibody responses.

MATERIALS AND METHODS

Virus Preparation

DEN and JE viral antigens were produced by inoculating DEN-2 (New Guinea C) or JE (Nakayama-NIH strain) virus into the brains of 1–3 day old suckling mice. After 5–7 days, the virus antigen was collected from infected mouse brain homogenate and further purified with sucrose-acetone. The purified antigens were titrated for hemagglutination titers, dispensed in small aliquots, and stored at −70°C until use.

Virology and Serology

Viral isolations for DEN viruses were attempted with serum samples from reported patients during the acute phase using C6/36 cell lines. The identification of the virus type was done by immunofluorescence assay using a panel of monoclonal antibodies against DEN and JE viruses. Diagnosis of DEN infections was based on virus isolation, hemagglutination inhibition (HI) test [Clarke and Casals, 1958], immunoglobulin M (IgM) enzyme-linked immunosorbent assay (ELISA) [Monath et al., 1984], and the polymerase-chain-reaction (PCR) method [Lanciotti et al., 1992], as described previously. A fourfold or greater rise in HI titer using paired acute and convalescent sera against DEN-1 or JE viral antigen was considered positive for acute DEN or JE infection, respectively. Sera collected through day 7 after the onset of symptoms are referred to as acute-phase samples. Convalescent sera refer to specimens that were collected 8 days or more after the onset of symptoms.

Human Serum Samples

The serum samples used in this study were collected from confirmed cases of DEN and JE patients reported to the National Institute of Preventive Medicine, Department of Health. Acute and/or convalescent sera from DEN patients were collected and analyzed. Convalescent sera from JE patients were analyzed for comparison. Patients were classified as primary DEN infections if a convalescent serum had an HI titer $\leq$1:1,280 against DEN-1 antigen or secondary if HI titer was >1:1,280 after the onset of illness [World Health Organization, 1986]. A clinical diagnosis of DHF was assigned following the World Health Organization clinical definition based on the presence of plasma leakage and thrombocytopenia [World Health Organization, 1986].

Epitope Prediction by Computer Software

The NS1 protein sequences used for epitope analysis in this study were derived from published amino acid sequences for the following flaviviruses: DEN-1 (Western Pacific) [Mason et al., 1987], DEN-2 (Jamaica) [Deubel et al., 1986], DEN-3 [Osatomi and Sumiyoshi, 1990], DEN-4 [Zhao et al., 1986], and JE virus (JaOArS982) [Sumiyoshi et al., 1987]. Protein sequence editing, analysis, and protein structure prediction were completed by using the DNASTAR Lasergene program (Windows version; DNASTAR Inc., Madison, WI). The sequences were aligned for maximum homology in order to determine the consensus sequence. The potential B-cell epitopes of DEN-2 NS1 protein were predicted and selected for peptide synthesis based on various analyses, including antigenic index, hydrophilicity, β-turn, surface probability, and flexibility. We have synthesized a series of 15-mer synthetic peptides from DEN-2 NS1 protein and used ELISA to screen immunodominant linear epitopes. An additional peptide, JE NS1-P1, derived from JE NS1 protein was synthesized to compare the binding specificity of sera from DEN and JE patients.

Peptide Synthesis

A series of peptides from DEN-2 NS1 sequences were synthesized on an automated multiple peptide synthesizer (model 396 MPS; Advanced Chemtech, Louisville, KY) by using F-moc chemistry on Wang resin. Peptides were cleaved from the resins by using trifluoroacetic acid, lyophilized, and dissolved in distilled water at a concentration of 1 mg/ml. The purity of each peptide

TABLE I. N-Terminal Amino Acid Sequences from the Nonstructural-1 Proteins of Dengue and Japanese Encephalitis Viruses

Virus	Amino acids	Amino acid sequence	Peptide[a]
D2	1–15	*N*-DSGCVVSWKNKELKC	D2 NS1-P1
D1	1–15	*N*-DSGCVINWKGRELKC	
D3	1–15	*N*-DMGCVINWKGKELKC	
D4	1–15	*N*-DMGCVVSWSGKELKC	
JE	1–15	*N*-DTGCAIDITRKEMRC	JE NS1-P1

[a]D2 NS1-P1, Dengue virus nonstructural-1 peptide; JE, Japanese encephalitis.

was monitored on C_{18} reverse-phase high-performance liquid chromatography and was found to be greater than 70%.

ELISA

ELISA was carried out as described previously with minor modification [Huang et al., 1996]. Briefly, each microtiter well (Immulon II; Dynatech Laboratories, Chantilly, VA) was coated overnight at 4°C with 10 μg/ml, 100 μl/well of synthetic peptides in 0.1 M carbonate buffer $Na_2CO_3/NaHCO_3$, pH 9.5. After washing with phosphate-buffered saline (PBS)-0.1% Tween 20 (PBST), wells were blocked with 200 μl of PBS-1% bovine serum albumin (BSA) for 1 hour at 37°C and incubated for 1 hour at 37°C with 100 μl of 1:100 diluted sera in PBST-1% BSA. Then, a 1:1,000 dilution of affinity-purified goat anti-human IgM plus IgG (IgM+IgG), anti-human IgM, or anti-human IgG conjugated to alkaline phosphatase (Jackson Immunochemicals, West Grove, PA) was added and incubated for 1 hour at 37°C. Finally, the enzyme activity was developed with the addition of substrate p-nitrophenylphosphate (Sigma, St. Louis, MO), and readings were taken at the dual wavelengths of 410 nm and 570 nm with a Dynatech MR700 microplate reader.

RESULTS

Epitope Mapping of DEN Virus NS1 Protein

To predict the immunodominant, linear B-cell epitopes, the amino acid sequences for the DEN-2 virus NS1 protein were analyzed by using the DNASTAR computer program. Based on the computer analyses, 19 15-mer peptides were synthesized and screened for ELISA reactivity with sera from DF patients. Ten convalescent sera from DF patients with high anti-DEN virus titer were selected to analyze peptide-specific IgM+IgG responses. Among the 19 peptides tested, one peptide D2 NS1-P1 (amino acids 1–15) located in the N-terminus was identified as the most immunodominant B-cell epitope that reacted with five convalescent sera tested (data not shown). An additional peptide, JE NS1-P1 (amino acids 1–15) derived from JE NS1 protein was synthesized for specificity tests. Table I shows the sequences of the D2 NS1-P1, JE NS1-P1, and homologous regions from D1, D3, and D4 NS1 proteins.

Reactivity of Immunodominant D2 NS1-P1 Peptide With Convalescent Sera From DF Patients and JE Patients

Once the immunodominant peptide (D2 NS1-P1) was identified, we analyzed the serospecificities of this peptide to convalescent sera from DF patients and JE patients. Sera from 20 DEN antibody-negative, normal adults were selected to determine the background binding. The cut-off value was calculated as the mean + 3 standard deviations and was set as 0.392. Figure 1 shows the representative data from convalescent sera of more than 50 DF patients. It was found that about 45% of the sera tested had significant IgM+IgG antibodies to D2 NS1-P1, whereas none of the sera from the 20 JE patients tested showed significant binding. To determine whether the D2 NS1-P1 peptide may represent a DEN-specific epitope, another peptide (JE NS1-P1; amino acids 1–15) from the homologous region of NS1 protein of the JE virus was synthesized. It is interesting to note that sera from DF patients, but not from JE patients, showed positive binding to JE NS1-P1 that was similar to that of D2 NS1-P1 (Fig. 1). It is tempting to speculate that JE patients do not respond to this N-terminal epitope of NS1 protein, whereas DEN patients produce cross-reactive antibodies due to high sequence conservation between D2 NS1-P1 and JE NS1-P1 (D2:DSGCV-KELKC and JE:DTGCA-KEMRC). For control, hepatitis C virus (HCV) C-P57, a peptide derived from HCV core protein, was used as a control peptide in the ELISA system. As expected, we did not observe nonspecific binding to HCV C-P57 in this assay system (data not shown).

Humoral IgM and IgG Responses of DF Patients to D2 NS1-P1

To determine the isotypes of antibodies specific to D2 NS1-P1, we analyzed the IgM and IgG antibodies from the DF patient sera that was positive for D2 NS1-P1 peptide. The isotype of D2 NS1-P1-specific antibodies was found to be mainly IgM in all sera that tested positive (Fig. 2). This result suggested that D2 NS1-P1 peptide represents an immunodominant, DEN-specific epitope that stimulates transient IgM antibody response.

Serotype Specificity and Dynamics of D2 NS1-P1-Specific Antibodies in Sera From Primary and Secondary DF Patients

To investigate the serotype-specificity and dynamics of D2 NS1-P1-specific antibodies in primary and secondary DF patients, we analyzed the IgM+IgG, IgM, and IgG responses from acute and/or convalescent sera from DF patients. Table II shows the results from selected DF patients (paired sera and four DEN serotypes) who had positive antibody responses to D2 NS1-P1 peptide. It was found that sera from all four DEN types bound to D2 NS1-P1 peptide. Dynamics studies on paired acute and convalescent sera from DF patients showed that IgM antibodies might be induced as

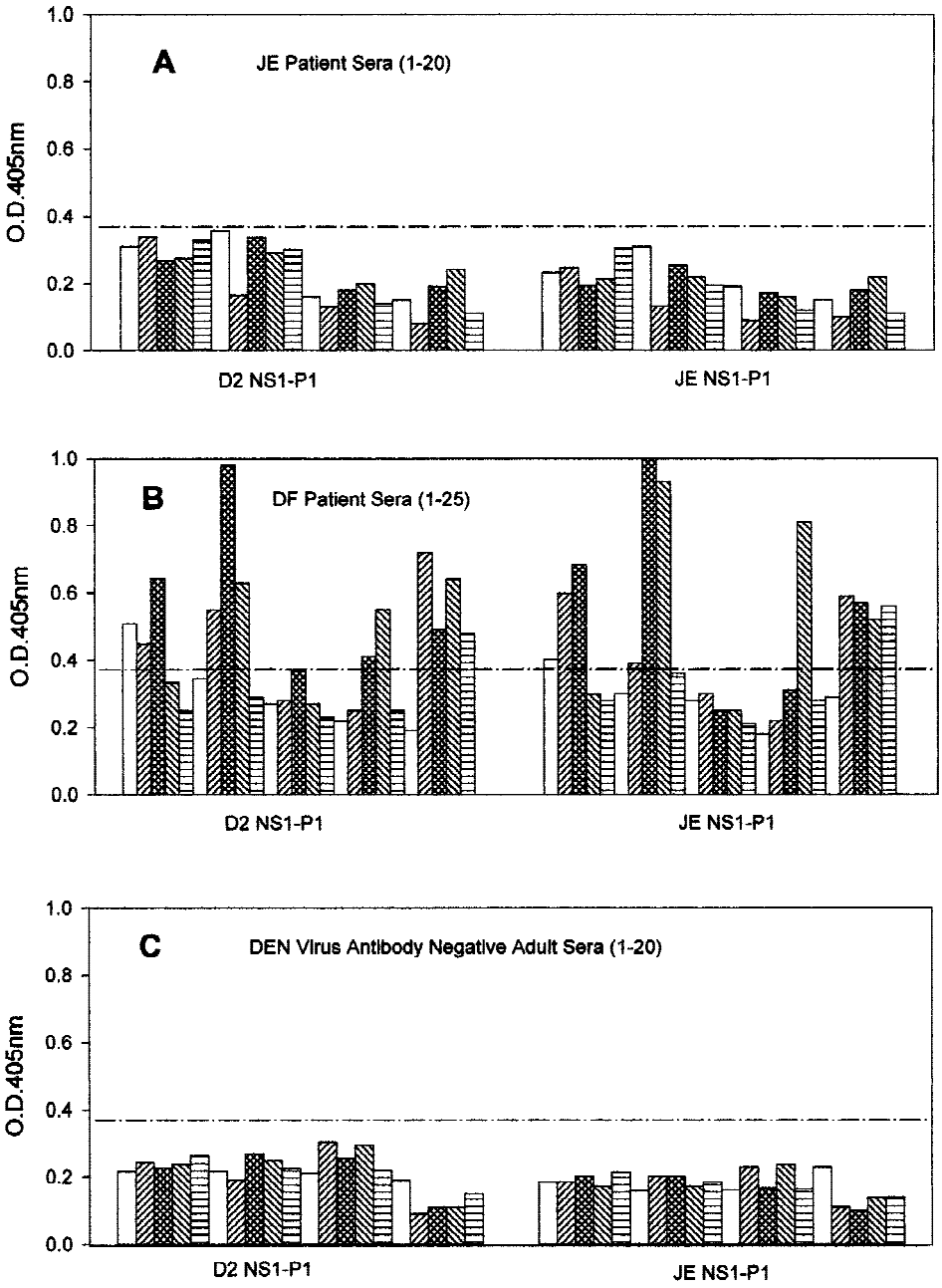

Fig. 1. Dengue (DEN) type 2 (D2) nonstructural-1 peptide (D2 NS1-P1)-specific and Japanese encephalitis (JE) NS1-P1-specific immunoglobulin M (IgM) plus IgG responses in convalescent sera of dengue fever (DF) patients and JE patients. Enzyme-linked immunosorbent assay (ELISA) was performed with sera from 20 JE patients **(A),** sera from 25 DF patients **(B),** and sera from 20 anti-DEN virus antibody-negative, normal adults **(C).** For further details, see Materials and Methods. O.D., optical density.

early as 2 days and remains detectable after 1 month. It is obvious from this study that significant anti-NS1 antibodies can be detected in DF patients with either primary or secondary infection. No correlation was observed between D2 NS1-P1-specific antibody titers and secondary infections. The results also showed that HI titers do not correlate well with D2 NS1-P1-specific antibody responses.

D2 NS1-P1-Specific Antibody Responses in Sera of DHF Patients

To determine whether DHF patients may have enhanced anti-NS1 antibodies and contribute to the immunopathogenesis of DHF, we analyzed the D2 NS1-P1-specific antibodies in the sera of DHF patients. Among seven DHF patient sera tested, three showed positive binding to D2 NS1-P1 peptide (Table III). Be-

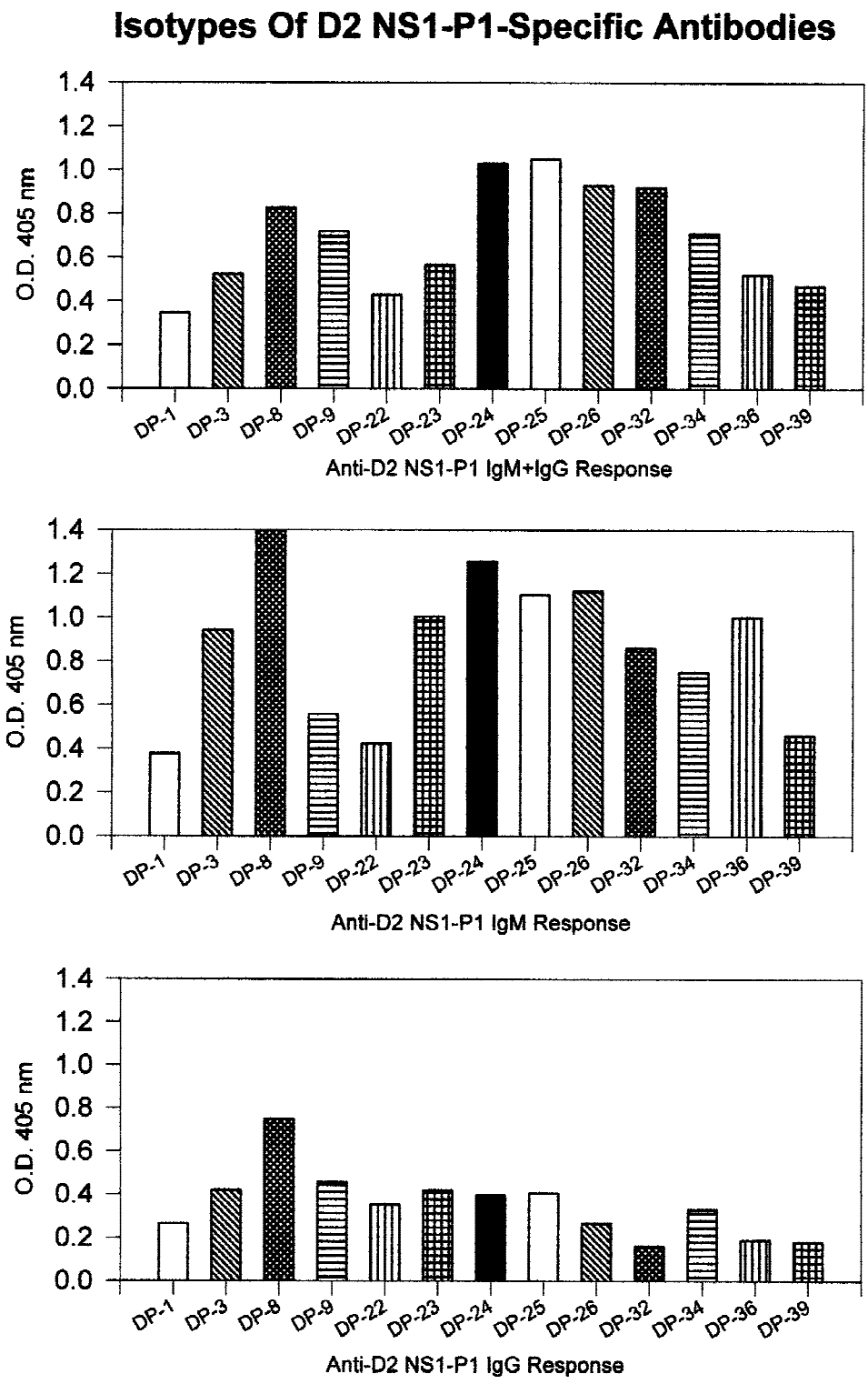

Fig. 2. Isotypes of D2 NS1-P1-specific antibodies in convalescent sera of DF patients. ELISA was performed with 13 sera of DF patients who were positive for D2 NS1-P1 peptide, as described in Materials and Methods.

cause four DHF patients did not have detectable anti-D2-NS1-P1 antibody, correlation between anti-NS1 antibody responses and DHF cannot be established from this study.

DISCUSSION

It is believed generally that ADE and viral virulence are two major factors contributing to the pathogenesis

TABLE II. Dengue Nonstructural-1 Peptide-Specific Antibody Responses from Dengue Fever Patients with Primary and Secondary Infections*

Dengue patient	Serotype	Type of infection	Status	Serum	HI titer	D2 NS1-P1 ELISA IgM+IgG	D2 NS1-P1 ELISA IgM	D2 NS1-P1 ELISA IgG
8600128	D1	Primary	DF	Acute (day 6)	< 10	0.681	0.461	0.357
				Convalescent (day 13)	160	0.718	0.614	0.322
8500846	D1	Primary	DF	Acute (day 7)	< 10	0.783	1.483	0.357
				Convalescent (day 14)	640	0.796	1.332	0.383
PL-034	D2	Primary	DF	Convalescent (day 37)	80	0.454	0.728	0.421
8600396	D2	?	DF	Acute (day 7)	1,280	0.435	0.450	0.286
8501051	D3	Primary	DF	Convalescent (day 12)	160	0.526	0.881	0.428
366325	D3	Secondary	DF	Acute (day 3)	< 10	0.413	0.539	0.330
				Convalescent (day 16)	2,560	0.455	0.452	0.286
466239	D4	Secondary	DF	Convalescent (day 22)	5,120	0.707	0.537	0.411
466127	D4	?	DF	Acute (day 4)	160	0.676	1.225	0.641
8600555	?	Primary	DF	Acute (day 2)	1,280	0.742	1.238	0.501
				Convalescent (day 10)	1,280	0.524	0.942	0.420
8600663	?	Secondary	DF	Acute (day 4)	2,560	0.491	0.971	0.294
				Convalescent (day 24)	10,240	0.560	0.794	0.386
8600518	?	Secondary	DF	Acute (day 7)	20	0.511	0.470	0.341
				Convalescent (day 14)	2,560	0.828	1.548	0.749
8600648	?	Secondary	DF	Acute (day 7)	2,560	0.502	0.493	0.323
				Convalescent (day 23)	1,280	0.430	0.433	0.353

*HI, hemagglutination inhibition; D2 NS1-P1, dengue nonstructural-1 peptide; ELISA, enzyme-linked immunosorbent assay; IgM, immunoglobulin M; IgG, immunoglobulin G; DF, dengue fever.

TABLE III. Dengue Nonstructural-1, Peptide-Specific Antibody Responses From Dengue Hemorrhagic Fever Patients

Dengue patient	Type of infection	Status	Serum	HI titer	D2 NS1-P1 ELISA IgM+IgG	D2 NS1-P1 ELISA IgM	D2 NS1-P1 ELISA IgG
8600324	Primary	DHF[a]	Convalescent (22 day)	1,280	0.302	0.290	0.260
8500529	Primary	DHF	Acute (6 day)	< 10	0.333	0.299	0.270
			Convalescent (15 day)	160	0.335	0.296	0.279
8500674	Secondary	DHF	Acute (4 day)	40	0.267	0.407	0.235
			Convalescent (14 day)	2,560	0.806	1.300	0.556
436021	Secondary	DHF	Convalescent (8 day)	5,120	0.524	0.820	0.339
415005	Secondary	DHF	Convalescent (32 day)	5,120	0.409	0.510	0.311
400024	Secondary	DHF	Convalescent (40 day)	5,120	0.223	0.185	0.236
424001	?	DHF	Acute (6 day)	640	0.317	0.211	0.280

[a]DHF, dengue hemorrhagic fever. For other abbreviations, see Table II.

of DHF. Although neutralizing antibodies to structural proteins of DEN virus has been shown to protect against homologous infection, it can also promote infection, depending on the concentration and the DEN virus encountered [Henchal et al., 1985, 1988]. To circumvent the problem of ADE, studies have been directed to NS proteins that might provide the protective immunity. Studies from passive and active immunizations have demonstrated protections against lethal DEN virus infections in mice with NS1 [Schlesinger et al., 1987; Falgout et al., 1990; Qu et al., 1993] and NS3 [Tan et al., 1990]. However, studies from anti-NS1 antibodies in primary and secondary DEN infections showed that anti-NS1 antibody responses were found almost exclusively during secondary infections [Falkler et al., 1973; Kuno et al., 1990; Churdboonchart et al., 1991]. This leads to the speculation that anti-NS1 antibody may promote infection and contribute to immunopathogenesis.

Recently, Falconar [1997] studied DEN virus NS1 antibody responses in mice by using monoclonal antibodies and synthetic peptides and reported that dimeric protein can generate antibodies that cross react with common epitopes on human blood clotting and integrin/adhesin proteins and binds to human endothelial cells. It was hypothesized that such reactions might account for the clinical findings in DHF/DSS. To better understand the immunological responses of infected individuals to various structural and NS proteins and their roles in protective immunity and/or pathogenesis of DHF, careful analyses using synthetic peptides and recombinant proteins are greatly needed.

In this study, we screened the continuous, B-cell epitopes on the DEN-2 NS1 protein by using synthetic peptides. A peptide, D2 NS1-P1 (amino acids 1–15), representing the immunodominant epitope, was identified that reacted with about 45% of convalescent sera from DF patients. The anti-D2 NS1-P1 antibody responses from DF patients can be summarized as follows: 1) Isotype analysis showed that most of the anti-D2 NS1-P1 antibodies belonged to IgM, although IgG could be induced in a few patients (Fig. 2, Table II). 2) Specificity study demonstrated that sera from all four DEN infections reacted to this peptide (Table II). 3) Dynamic studies showed that IgM antibodies can be detected as early as day 2 after the onset of symptom

(Table II). 4) Similar antibody responses to D2 NS1-P1 were observed for primary and secondary DF patients (Table II). No correlation can be established between anti-D2 NS1-P1 antibody responses and DHF, because sera from DF and DHF patients showed similar antibody responses.

This study provides new insight concerning the anti-NS1 antibody responses raised by DEN patients. It is interesting to compare the anti-NS1 antibodies measured by Western blot, complement fixation, and peptide-ELISA from various reports. Kuno et al. [1990] and Churdboonchart et al. [1991] determined anti-NS1 antibodies in acute and convalescent serum from patients with DEN infections by using Western blots. They identified IgG reactive with E-glycoprotein and two NS proteins, NS3 and NS5, but not NS1, in convalescent serum from patients with primary DEN infection. In convalescent serum from patients with secondary DEN infection, they can detect antibodies to E-glycoprotein, C, pre-M, NS1, NS3, NS4a, and NS5 proteins. A similar result was reported by Falkler et al. [1973] by using complement fixation test. We are able to detect D2 NS1-P1-specific IgM responses in about 45% of sera from DF patients that have either primary or secondary infections. To our knowledge, this is the first demonstration that significant anti-NS1 antibodies can be detected in the sera of primary dengue infection. Because conformational epitopes on native, dimeric NS1 protein may be important in inducing secondary IgG antibodies, further analyses using native or recombinant NS1 protein may lead to more rewarding results.

REFERENCES

Bhamarapravati N. 1989. Hemostatic defects in dengue hemorrhagic fever. J Infect Dis 4(Suppl.):S826–S829.

Bravo JR, Guzman MG, Kouri GP. 1987. Why dengue haemorrhagic fever in Cuba? I. Individual risk factors for dengue haemorrhagic fever/dengue shock syndrome (DHF/DSS). Trans R Soc Trop Med Hygiene 81:816–820.

Burke DS, Nisalak A, Johnson DE, Scott RM. 1988. A prospective study of dengue infections in Bangkok. Am J Trop Med Hygiene 38:172–180.

Churdboonchart V, Bhamarapravati N, Peampramprecha S, Sirinavin S. 1991. Antibodies against dengue viral proteins in primary and secondary dengue hemorrhagic fever. Am J Trop Med Hygiene 44:481–493.

Clarke DH, Casals J. 1958. Techniques for hemagglutination and hemagglutination inhibition with arthropod-borne virus. Am J Trop Med Hygiene 7:561–577.

Deubel V, Kinney RM, Trent DW. 1986. Nucleotide sequence and deduced amino acid sequence of the structural proteins of dengue type 2 virus, Jamaica genotype. Virology 155:365–377.

Falconar AK. 1997. The dengue virus nonstructural-1 protein (NS1) generates antibodies to common epitopes on human blood clotting, integrin/adhesin proteins and binds to human endothelial cells: potential implications in haemorrhagic fever pathogenesis. Arch Virol 142:897–916.

Falgout B, Bray M, Schlesinger JJ, Lai CJ. 1990. Immunization of mice with recombinant vaccinia virus expressing authentic dengue virus nonstructural protein NS1 protects against lethal dengue virus encephalitis. J Virol 64:4356–4363.

Falkler WA, Diwan AR, Halstead SB. 1973. Human antibody to dengue soluble complement-fixing (SCF) antigens. J Immunol 111: 1804–1809.

Gubler DJ. 1997. Dengue and dengue hemorrhagic fever: its history and resurgence as a global public health problem. In Gubler DJ, Kuno G, editors. Dengue and dengue hemorrhagic fever. New York: CAB International, p 1–22.

Gubler DJ, Reed D, Rosen L, Hitchcock JCJ. 1978. Epidemiologic, clinical, and virologic observations on dengue in the kingdom of Tonga. Am J Trop Med Hygiene 27:581–589.

Halstead SB. 1970. Observations related to pathogenesis of dengue hemorrhagic fever. VI. Hypotheses and discussion. Yale J Biol Med 42:350–362.

Halstead SB. 1979. In vivo enhancement of dengue virus infection in rhesus monkeys by passively transferred antibody. J Infect Dis 140:527–533.

Halstead SB, O'Rourke EJ. 1977. Dengue viruses and mononuclear phagocytes. I. Infection enhancement by non-neutralizing antibody. J Exp Med 146:201–217.

Halstead SB, Shotwell H, Casals J. 1973. Studies on the pathogenesis of dengue infection in monkeys II. Clinical laboratory responses to heterologous infection. J Infect Dis 128:15–22.

Henchal EA, McCown JM, Burke DS, Seguin MC, Brandt WE. 1985. Epitopic analysis of antigenic determinants on the surface of dengue-2 virus using monoclonal antibodies. Am J Trop Med Hygiene 34:162–169.

Henchal EA, Henchal LS, Schlesinger JJ. 1988. Synergistic interactions of anti-NS1 monoclonal antibodies protect passively immunized mice from lethal challenge with dengue 2 virus. J Gen Virol 69:2101–2107.

Huang JH, Wey JJ, Lee HF, Tsou TL, Wu CS, Wu JR, Chen HM, Chin C, Chien LJ, Chen LK, Wu YC, Pan MJ, Wang TM. 1996. Identification of immunodominant, group-specific and subcomplex-specific, continuous epitopes in the core regions of Japanese encephalitis virus using synthetic peptides. Virus Res 41:43–53.

Kuno G, Vorndam AV, Gubler DJ, Gomez I. 1990. Study of anti-dengue NS1 antibody by Western blot. J Med Virol 32:102–108.

Lanciotti, RS, Calisher, CH, Gubler DJ, Chang GJ, Vorndam AV. 1992. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. J Clin Microbiol 30:545–551.

Mady BJ, Kurane I, Erbe DV, Fanger MW, Ennis FA. 1993. Neuraminidase augments Fc receptor II-mediated antibody-dependent enhancement of dengue virus infection. J Gen Virol 74:839–844.

Mason PW, McAda PC, Mason TL, Fournier MJ. 1987. Sequence of the dengue-1 virus genome in the region encoding the three structural proteins and the major nonstructural protein NS1. Virology 161:262–267.

Monath TP, Nystrom RR, Bailey RE, Calisher CH, Muth DJ. 1984. Immunoglobulin M antibody capture enzyme-linked immunosorbent assay for diagnosis of St. Louis encephalitis. J Clin Microbiol 20:784–790.

Nimmannitya S. 1987. Clinical spectrum and management of dengue haemorrhagic fever. Southeast Asian J Trop Med Public Health 18:392–397.

Nimmannitya S, Halstead SB, Cohen SN, Margiotta MR. 1969. Dengue and Chikungunya virus infection in man in Thailand, 1962–1964. I. Observations on hospitalized patients with haemorrhagic fever. Am J Trop Med Hygiene 18:954–971.

Osatomi K, Sumiyoshi H. 1990. Complete nucleotide sequence of dengue type 3 virus genome RNA. Virology 176:643–647.

Qu X, Chen W, Maguire T, Austin F. 1993. Immunoreactivity and protective effects in mice of a recombinant dengue 2 Tonga virus NS1 protein produced in baculovirus expression system. J Gen Virol 74:89–97.

Rosen L. 1977. The Emperor's New Clothes revisited, or reflections on the pathogenesis of dengue hemorrhagic fever. Am J Trop Med Hygiene 26:337–343.

Russell PK, Nisalak A, Sukhavachana P, Vivona S. 1967. A plaque reduction test for dengue virus neutralizing antibodies. J Immunol 99:285–290.

Sangkawibha N, Rojanasuphot S, Ahandrik S, Viriyapongse S, Jatanasen S, Salitul V, Phanthumachinda B, Halstead SB. 1984. Risk factors for dengue shock syndrome: a prospective epidemiologic study in Rayong, Thailand. I. The 1980 outbreak. Am J Epidemiol 120:653–669.

Schlesinger JJ, Brandriss MW, Walsh EE. 1987. Protection of mice against dengue 2 virus encephalitis by immunization with the dengue 2 virus non-structural glycoprotein NS1. J Gen Virol 68: 853–857.

Sumiyoshi H, Mori C, Fuke I, Morita K, Kuhara S, Kondou J, Kikuchi Y, Nagamatu H, Igarashi A. 1987. Complete nucleotide sequence of the Japanese encephalitis virus genome RNA. Virology 161: 497–510.

Tan CHC, Yap EH, Singh M, Deubel V, Chan YC. 1990. Passive protection studies in mice with monoclonal antibodies directed against the non-structural protein NS3 of dengue 1 virus. J Gen Virol 71:745–748.

World Health Organization. 1986. Dengue haemorrhagic fever: diagnosis, treatment and control. Geneva: World Health Organization.

World Health Organization. 1994. Dengue haemorrhagic fever: diagnosis, treatment and control. Geneva: World Health Organization.

Zhao B, Mackow E, Buckler-White A, Markoff L, Chanock RM, Lai CJ, Makino Y. 1986. Cloning full-length dengue type 4 DNA sequences: analysis of genes coding for structural proteins. Virology 155:77–88.